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The rat suprachiasmatic nucleus is a clock for all seasons

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ABSTRACT **Seasonal changes of daylength (photoperiod) affect the expression of hormonal and behavioral circadian rhythms in a variety of organisms. In mammals, such effects might reflect photoperiodic changes in the circadian pacemaking system [located in the suprachiasmatic nucleus (SCN) of the hypothalamus] that governs these rhythms, but to date no functionally relevant, intrinsic property of the SCN has been shown to be photoperiod dependent. We have analyzed the temporal regulation of light-induced c-*fos* gene expression in the SCN of rats maintained in long or short photoperiods. Both *in situ* hybridization and immunohistochemical assays show that the endogenous circadian rhythm of light responsiveness in the SCN is altered by photoperiod, with the duration of the photosensitive subjective night under the short photoperiod 5–6 h longer than under the long photoperiod. Our results provide evidence that a functional property of the SCN is altered by photoperiod and suggest that the nucleus is involved in photoperiodic time measurement.**

Over the course of the year, most mammals respond to seasonal changes of daylength (photoperiod) with altered physiology and behavior. Photoperiodic information from the environment is conveyed to the organism by a circadian rhythm of melatonin production in the pineal gland (1–4). In the rat, rhythmic melatonin production is driven by a circadian rhythm of the activity of pineal *N*-acetyltransferase (NAT), which synthesizes the melatonin precursor *N*-acetylserotonin (5, 6). The NAT rhythm is controlled by a light-entrainable circadian pacemaking system in the suprachiasmatic nucleus (SCN) of the hypothalamus; SCN lesions abolish this rhythm (7). Norepinephrine released at night from sympathetic nerve endings in the pineal gland stimulates adrenergic receptors and the cAMP pathway; the resulting induction and activation of pineal NAT activity leads to high nighttime melatonin levels (8). In the rat, the NAT rhythm is entrained to the 24-h light–dark (LD) cycle primarily by light onset at dawn (9). With longer daylengths, light at an earlier dawn advances the phase of the morning NAT decline, while light at a later dusk delays the phase of the evening NAT rise (3, 10). The resulting alteration in the duration of the nocturnal melatonin signal, compressed during long summer days and decompressed during short winter days, appears to serve as an endogenous photoperiodic message (2, 3, 11).

When rats and hamsters are transferred from a long to a short photoperiod, the decompression of the melatonin signal occurs gradually (12–14). Since high NAT activity can be induced independent of the photoperiod by administration of a β-adrenergic agonist (15), it seems likely that the neural substrate for this gradual decompression lies upstream of the pineal gland, perhaps in the SCN. Importantly, another circadian rhythm governed by the SCN is photoperiodic. The duration of nocturnal locomotor activity (α) in hamsters is also compressed during long days and decompressed during short days (16, 17); like melatonin, α decompresses only gradually after animals are transferred from a long to a short photoperiod (17). These parallel changes in the waveforms of two SCN-dependent rhythms suggest that the functional state of the circadian pacemaking system driving them is likely to depend on photoperiod.

A property that has proven useful as an *in vivo* marker of SCN photic sensitivity and intrinsic rhythmicity is the light-induced stimulation of immediate-early genes (for reviews, see refs. 18 and 19). The most intensively studied of these, c-*fos*, encodes a sequence-specific DNA-binding protein that alters the expression of target genes by regulating their transcription. Levels of c-*fos* mRNA and immunoreactive Fos protein in the retinorecipient zone of the SCN are dramatically elevated (from essentially undetectable levels) by light. This photic stimulation depends on circadian phase, with a phase–response relationship similar to that for light-induced phase shifts of locomotor rhythmicity. In constant darkness, light pulses administered during the subjective night evoke c-*fos* (as well as phase shifts of behavioral rhythmicity), whereas light pulses delivered during the subjective day elicit neither c-*fos* nor phase shifts. The fact that the photic induction of c-*fos* in the SCN depends on circadian phase means that the expression of c-*fos* is clock-controlled.

In this paper, we determined whether photoperiod affects the mechanism for phase-dependent photic induction of c-*fos* in the SCN. Rats were maintained under long (LD 16:8) or short (LD 8:16) photoperiods, and light-induced c-*fos* mRNA and Fos protein expression were measured at different time points by using *in situ* hybridization and immunohistochemistry, respectively. The results demonstrate that the state of the circadian pacemaking system in the rat SCN is changed as a function of photoperiod. We found that the duration of the endogenous interval that enables c-*fos* photoinduction under short days is 5–6 h longer than the interval under long days.

MATERIALS AND METHODS

Animals. Sixty-day-old male Wistar rats (Velaz, Prague) were housed at a temperature of 23°C ± 2°C with free access to food and water. Animals were maintained under either a long photoperiod (LD 16:8, lights on from 04:00 to 20:00) or a short photoperiod (LD 8:16, lights on from 0800 to 1600) for at least 3 weeks prior to experiments. Illumination intensity provided by overhead 40 W fluorescent tubes (Tesla, Prague) was between 50 and 200 lux, depending on cage position.

Experimental Paradigm. At various time points, rats were exposed to a single 30-min, ≈200-lux light pulse and killed at the end of the pulse (for c-*fos* mRNA), returned to darkness and killed 30 min later (for Fos immunoreactivity), or returned to darkness and killed the next day (for phase shifts of the NAT rhythm; data to be reported elsewhere). Fig. 1 illustrates how masking effects of the entraining LD cycles were avoided. When evening and early night time points were studied, the onset of darkness was advanced to 1200 from the usual 2000 in LD 16:8 and 1600 in LD 8:16; when late night and morning

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Abbreviations: SCN, suprachiasmatic nucleus; NAT, pineal *N*-acetyltransferase; LD, light–dark.

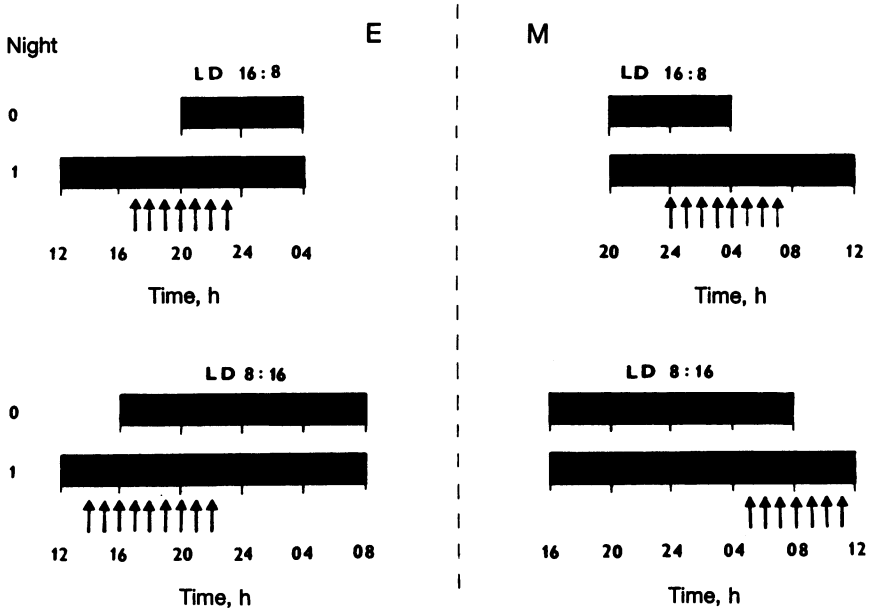

FIG. 1. Experimental paradigm. Rats were maintained in either LD 16:8 or LD 8:16 prior to experiments (Night 0). On the day of experiment (Night 1), the onset of darkness was advanced to 1200 (E, evening) or the onset of light was delayed to 1200 (M, morning), and rats were exposed to a single 30-min light pulse at the times indicated by the arrows. Bars represent environmental darkness.

time points were studied, the onset of light was delayed to 1200 from the usual 0400 in LD 16:8 and 0800 in LD 8:16. Control rats received no light pulse and were killed quickly under dim red light.

***In Situ* Hybridization.** Rats were decapitated (guillotine), and brains were rapidly removed, frozen in 2-methylbutane cooled to −25°C with dry ice, and stored at −80°C. Coronal 20-μm thick sections were cut on a cryostat, mounted onto slides subbed with Vectabond (Vector Laboratories), and stored at −80°C. Sections were fixed in 3% paraformaldehyde in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) for 7 min, washed two times in 2× SSC for 10 min, treated with 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride for 10 min, dehydrated with alcohol, and incubated in prehybridization buffer (5× SSC/5× Denhardt's solution (1× Denhardt's = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/50% formamide/0.1% SDS/50 mM dithiothreitol) for 1 h at 42°C followed by buffer containing 0.8 × 10^6 cpm of ^{35}S-labeled c-*fos* cDNA, 10% dextran sulfate, 1 mM dNTP, 200 μg of salmon sperm DNA per ml, and 0.1% $Na_4P_2O_7$ overnight at 42°C. The sections were then washed in 2× SSC two times for 30 min at 22°C and two times for 30 min at 42°C; in 0.5× SSC two times for 30 min at 22°C; and in 0.1× SSC two times for 30 min at 22°C, two times for 30 min at 42°C, and one time for 30 min at 22°C. Slides were dehydrated in alcohol containing 300 mM ammonium acetate, dried, and exposed to Hyperfilm-MP (Amersham) for 72–96 h. The OD of the autoradiographic hybridization signal was measured by using a Zeiss (Kontron, Zurich) Image Processing System. The average OD for each rat was derived from at least two sections through the mid to caudal SCN, each expressed as a relative OD (ratio of SCN OD/OD surrounding hypothalamus).

The c-*fos* cDNA probe was derived from a 2.2-kb rat cDNA inserted into the *Eco*RI restriction site of pSP65 (ref. 20; generously supplied by Tom Curran, Roche Institute of Molecular Biology, Nutley, NJ). It was prepared by digesting the cDNA with *Dde* I, retrieving the fragments by extraction with phenol/chloroform extraction and precipitation with ethanol, and labeling with [^{35}S]dCTP by the random-primed method (Boehringer Mannheim). Labeled probe was isolated by passage through a Sephadex G-25 spin column (Boehringer Mannheim).

Immunohistochemistry. Rats were deeply anesthetized with pentobarbital (50 mg i.p.) and perfused through the ascending aorta with heparinized saline followed by phosphate-buffered saline (PBS; 0.01 M sodium phosphate/0.15 M NaCl, pH 7.2) and then freshly prepared 4% paraformaldehyde in PBS. Brains were removed, postfixed for 12 h at 4°C, and cryoprotected in 20% sucrose in PBS overnight at 4°C. Coronal 30-μm thick sections were cut and processed for immunohistochemistry by using the avidin/biotin method with diaminobenzidine as the chromogen, as described (21). The primary Fos antiserum (1:8000) was generated against the N-terminal peptide sequence (kindly provided by D. Hancock; Imperial Cancer Research Fund, London and generously supplied by Michael Hastings, University of Cambridge, U.K.). Labeled cell nuclei in the ventrolateral SCN (irrespective of the intensity of staining) were counted in the most heavily stained section by two independent observers without knowledge of the experimental procedure.

Statistical Analysis. Log transformed data were analyzed by one-way analysis of variance and subsequent pairwise comparisons by the Bonferroni *t* statistic.

RESULTS

SCN c-*fos* mRNA Induction Under Long and Short Photoperiods. No autoradiographic hybridization signal was detectable in the SCN of control rats killed in darkness in the evening or the morning (relative mean OD ± SEM was 1.32 ± 0.06; $n = 7$).

In rats maintained in LD 16:8 and exposed to evening and early night light pulses, the earliest time point with significantly increased c-*fos* mRNA induction was 2100 ($P > 0.05$, compared with 1800)—i.e., 1 h after the usual dark onset at 2000 in this long photoperiod. In rats maintained in LD 8:16, the earliest time point was at 1900 ($P < 0.001$, compared with 1600 or 1700)—i.e., 3 h after the usual dark onset at 1600 in this short photoperiod. Thus, c-*fos* mRNA levels in the SCN were increased by an evening light pulse 2 h earlier in rats maintained in the short photoperiod than in those maintained in the long one (Fig. 2*A Left*).

In rats maintained in LD 16:8 and exposed to late night and morning light pulses, the earliest time point with significantly decreased c-*fos* mRNA induction was 0400 ($P < 0.01$, compared to 0300)—i.e., at the usual light onset in this long photoperiod. Induction was decreased further at 0500 ($P < 0.05$) and remained at this low level at 0600 and 0700. In rats maintained in LD 8:16, the earliest time point was at 0800 (*P*

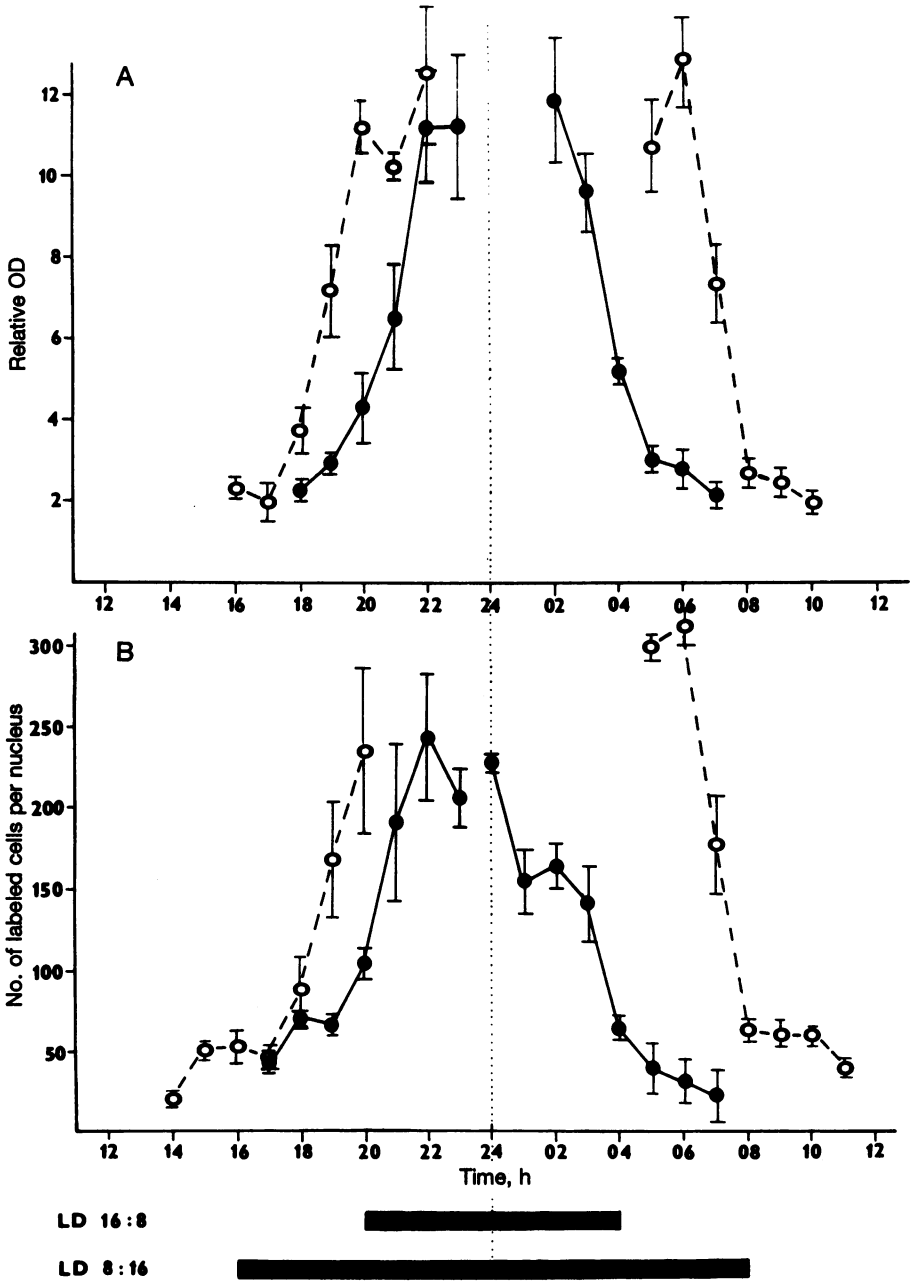

FIG. 2. Light-induced c-*fos* gene expression in the SCN under long and short photoperiods. Phase-dependent photic induction of c-*fos* mRNA (*A; in situ* hybridization) and Fos protein (*B*; immunohistochemistry) in the SCN of rats previously maintained in either a long (LD 16:8, ●) or a short (LD 8:16, ○) photoperiod and exposed to a single 30-min light pulse around the time of the original light–dark and dark–light transitions. Each point represents the mean ± SEM from 4–8 (*in situ* hybridization) or 2–8 (immunohistochemistry) animals.

< 0.001, compared with 0500 or 0600)—i.e., at the usual light onset in this short photoperiod. Thus, c-*fos* mRNA levels in the SCN induced by a morning light pulse were decreased 4 h later in rats maintained in the short photoperiod than in those maintained in the long one (Fig. 2*A Right*).

These results show that the duration of the endogenous interval that enables c-*fos* (mRNA) photoinduction under short days is about 6 h wider than under long days.

SCN Fos Protein Expression Under Long and Short Photoperiods. Fos immunoreactivity was barely detectable in the SCN of control rats killed in darkness in the evening or the morning (mean number of labeled cells per nucleus ± SEM was 4.4 ± 1.4; $n = 25$).

In rats maintained in LD 16:8 and exposed to evening and early night light pulses, the earliest time points with significantly increased Fos protein expression were 2000 ($P < 0.01$, compared with 1700) and 2100 ($P < 0.01$, compared with 18:00)—i.e., about 1 h after the usual dark onset at 2000 in this long photoperiod. In rats maintained in LD 8:16, the earliest time point was at 1900 ($P < 0.01$, compared with 1700)—i.e., 3 h after the usual dark onset at 1600 in this short photoperiod. Thus, Fos protein levels in the SCN were increased by an evening light pulse about 2 h earlier in rats maintained in the short photoperiod than in those maintained in the long one (Fig. 2*B Left*).

In rats maintained in LD 16:8 and exposed to late night and morning light pulses, the earliest time point with significantly decreased Fos protein expression was 0400 ($P < 0.01$, compared with 0300)—i.e., at the usual light onset in this long photoperiod. In rats maintained in LD 8:16, the earliest time point was at 0700 ($P < 0.05$, compared with 0600)—i.e., 1 h before the usual light onset at 0800 in this short photoperiod. Expression was decreased further at 0800 ($P < 0.001$). Thus, Fos protein levels in the SCN induced by a morning light pulse were decreased 3 h later in rats maintained in the short photoperiod than in those maintained in the long one (Fig. 2*B Right*).

These results show that the duration of the endogenous interval that enables Fos (protein) photic expression under short days is about 5 h wider than under long days.

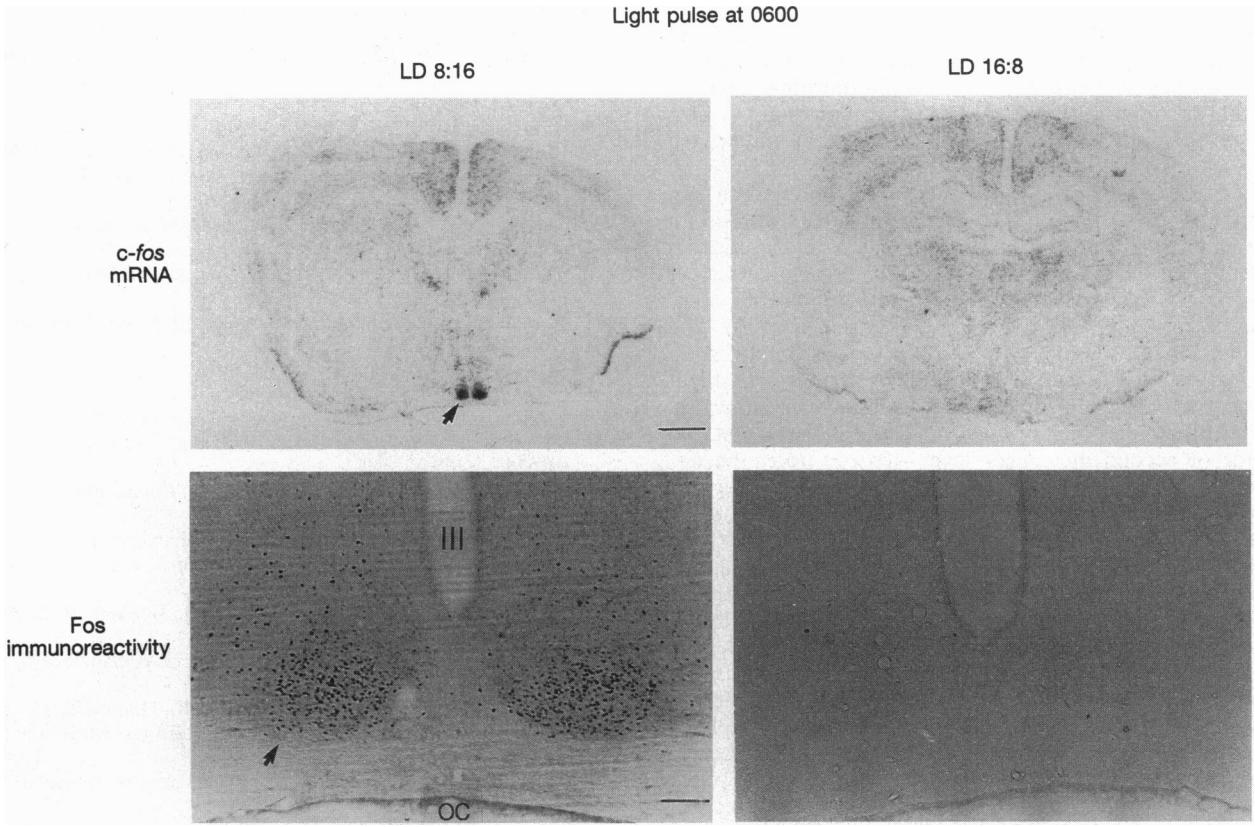

FIG. 3. Light-induced SCN c-*fos* gene expression in the morning. After a 30-min light pulse at 0600 in rats maintained in LD 8:16 (*Left*), levels of c-*fos* mRNA (*in situ* hybridization, whole-tissue section) and Fos protein (immunohistochemistry, SCN region) were expressed maximally in the SCN (arrows); at the same time point in LD 16:8 (*Right*), no stimulation was detected. III, third ventricle; OC, optic chiasm. Scale bars represent 1 mm (*Upper*) and 100 μm (*Lower*).

Asymmetric Expansion of the Endogenous Interval Under the Short Photoperiod. The foregoing data indicate that the interval enabling c-*fos* photoinduction in the SCN widens unevenly from long LD 16:8 days to short LD 8:16 days. Induction was advanced 2 h earlier in the evening but persisted 3–4 h later in the morning. Thus, the most dramatic photoperiodic difference in c-*fos* inducibility was seen in the morning. At 0600 in rats maintained in LD 8:16, c-*fos* mRNA and Fos protein expression were maximum; at the same time point in LD 16:8, no stimulation was detected (Fig. 3).

DISCUSSION

Our findings demonstrate that the endogenous rhythm of light responsiveness in the rat SCN is dependent on photoperiod. Both *in situ* hybridization and immunohistochemical assays of c-*fos* gene expression show that the duration of the photosensitive subjective night under short days is nearly double the duration under long days. This result implies that the functional state of the circadian pacemaking system in the SCN is altered by photoperiod. Thus, photoperiodic changes in the nocturnal duration of melatonin production and locomotor activity—compressed during long days and decompressed during short days—are likely to reflect changes in the SCN itself, suggesting that the nucleus is involved in processing photoperiodic information from the environment. Although the rat is only marginally photoperiodic (22, 23), it may be sensitized to the effects of photoperiod by neonatal treatment with testosterone propionate (24), olfactory bulbectomy (25), or undernutrition (26).

Our data are in accordance with previous observations that the circadian phase–response curves for photic phase shifts of the rat NAT (9, 27) and hamster locomotor (‡; J. A. Elliot and C. S. Pittendrigh, personal communication) rhythms depend on the prior photoperiod. Until now, seasonal variations of vasopressin immunoreactivity have been the only reported changes in the SCN over the course of a year (29, 30). While it appears likely that the pattern of spontaneous neuronal discharge of single SCN cells is also affected by photoperiod (31), there is no direct evidence on this point.

It is noteworthy that the endogenous interval enabling c-*fos* photoinduction in the rat SCN extends more into the morning than into the evening, when the photoperiod shortens. This observation has also been made for the waveform (3) and photic phase shifts of the rhythm of pineal NAT activity. A light stimulus administered at the end of the dark phase shifts the NAT rhythm under both long and short photoperiods, whereas a light stimulus administered at the beginning of the dark phase shifts the rhythm only under a long photoperiod (9, 10). These findings underscore the importance of the morning light onset at dawn for entrainment of the rat circadian system to the external LD cycle; the evening light offset at dusk may serve as a photoperiodic signal. The correlation between the time courses of c-*fos* photoinduction in the SCN and melatonin production in the pineal suggests that the duration of the nocturnal melatonin signal under various photoperiods is shaped, at least in the rat, by the entraining rather than the suppressive effects of light (32).

Among other possibilities, a photoperiodic clock in the SCN might be explained by a model of a complex circadian pacemaker consisting of evening and morning components (33). The phase relationship between the two components would

‡Elliot, J. A., Fourth Meeting of the Society for Research in Biological Rhythms, May 4–8, 1994, Amelia Island, FL, p. 127 (abstr).

depend on the photoperiod, compressed during long days and decompressed during short days. Independent phase shifts of the evening rise and morning fall of nocturnal NAT activity (3, 10, 34), as well as the evening onset and morning offset of nocturnal locomotor activity (17, 35) are consistent with a complex evening–morning pacemaking system underlying these rhythms.

We expect that the changes we observed in immunoreactive Fos protein levels were due to changes in Fos synthesis. In studies of intact brain, *in situ* hybridization has been used as an index of c-*fos* gene transcription, although actual nuclear run-on assays have been reported only for the osmotic stimulation of c-*fos* mRNA in the supraoptic nucleus (36). The phase-dependent, photoperiodic mechanism that enables the photic induction of c-*fos* in the SCN is unknown. It might operate very proximally in the signal-transduction pathway—e.g., in the retina—and/or within SCN cells—e.g., at the receptor or second messenger level—and/or upon the cis-acting regulatory DNA sequences of the c-*fos* gene. Significantly, the mechanism appears to be specific to the SCN, since photic stimulation of c-*fos* mRNA and immunoreactive Fos protein levels in the rat intergeniculate leaflet does not depend on the time of day of stimulation (37). It may also be specific to photic stimulation, since activation of c-*fos* mRNA by D_1 dopamine receptors in the fetal rat SCN is also independent of the phase of stimulation (38). Importantly, circadian control of c-*fos* expression persists in the SCN *in vitro*; electrical stimulation of the optic nerves induces c-*fos* mRNA in SCN explants only during the subjective night (39). Thus, neither the retina nor the rest of the brain is a required constituent for the phase dependency of c-*fos* induction (although they might affect it). Since the electrophysiological responsiveness of SCN cells to light does not change as a function of circadian phase (40), the mechanism for circadian control of Fos expression may be at the level of intracellular signal-transduction pathways.

Our understanding of the circadian organization of seasonal timekeeping is advancing. For example, in *Drosophila melanogaster* (28), as well as in *Drosophila auraria* (C. S. Pittendrigh, personal communication), the photoperiodic time measurement underlying the seasonality of diapause appears to be made by a circadian oscillation that is distinct from the circadian oscillation controlling locomotion and eclosion. In the rat, we have found that the discrete brain nucleus that governs circadian locomotor and hormonal rhythms has an endogenous rhythm of light responsiveness that depends on photoperiod. The circadian clock in the SCN appears to be a clock for all seasons (33), regulating photoperiodic changes in circadian rhythmicity, physiology, and behavior.

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